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ORIGIN OF HAWKSBILL TURTLES IN A CARIBBEAN FEEDING AREA AS INDICATED BY GENETIC MARKERS[1]

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Abstract. Hawksbill turtles move between nesting colonies and feeding grounds, but in most cases it is not known which reproductive populations occupy a particular feeding habitat. In this study, genetic markers derived from mitochondrial DNA sequences are used to estimate the contribution of Caribbean nesting colonies to a feeding ground at Mona Island, Puerto Rico (n = 41). Maximum likelihood analysis indicates that this feeding population is not composed primarily of turtles from the neighboring nesting colony (also on Mona Island), but is drawn from nesting populations throughout the Caribbean region. A sampled nesting colony in the southern hemisphere (Bahia, Brazil) did not contribute, at detectable levels, to the Mona Island feeding ground. From this evidence, we concluded that hawksbill turtles recruit to feeding grounds over a scale of hundreds of kilometres, but not over the scale of 7000 km that separate Mona Island from Bahia, Brazil. These data indicate that a hawksbill turtle harvest on feeding grounds will reduce nesting populations throughout the Caribbean region.

Key words: Caribbean Sea; conservation genetics; DNA sequencing; demography; Eretmochelys imbricata; *feeding ground composition; genetic markers; marine turtles; mitochondrial DNA; mixed stock analysis; population structure.*

INTRODUCTION

Marine turtles are difficult to observe in foraging habitat and, for this reason, several components of their life history remain obscure. Most marine turtle species are believed to occupy a pelagic habitat after hatching, and to recruit eventually to juvenile feeding grounds along coastal margins (Carr 1987). However, it is not generally known which nesting areas contribute to a particular feeding area, and this gap hampers a complete understanding of marine turtle life history (Limpus 1992, Limpus et al. 1992). Since marine turtles continue to be harvested on foraging areas, this facet of their natural history also has prominent conservation implications. When a hawksbill turtle (*Eretmochelys imbricata*) is captured on a feeding area, does this activity impact a neighboring rookery or more distant

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nesting populations? Establishing which nesting populations contribute to a particular feeding area (i.e., a demographic link between nesting aggregates and feeding aggregates) can indicate which breeding populations are affected by commercial fisheries and other human incursions.

One relatively new approach to estimating the origin of migratory cohorts or mixed stocks involves the use of natural genetic markers (Avise 1994). To resolve the complex stock structure of anadromous salmon in marine fisheries, researchers developed a maximum likelihood (ML) model to estimate the contribution of multiple freshwater spawning grounds to coastal and oceanic fisheries (Grant et al. 1980, Millar 1987, Pella and Milner 1987). ML estimates are typically based on differences in allele frequencies among riverine stocks, as detected by protein electrophoresis.

In principle, this approach is applicable to the analysis of marine turtle feeding areas and migratory corridors. Nesting-beach surveys demonstrate the presence of rookery-specific mitochondrial (mt) DNA haplotypes in green turtles (*Chelonia mydas*; Bowen et al. 1992, Norman et al. 1994, Encalada 1995), loggerhead turtles (*Caretta caretta*; Bowen et al. 1993, 1994), and hawksbill turtles (Broderick et al. 1994, Bass et al. 1996). These "endemic" haplotypes and related haplotype frequency shifts among nesting colonies may provide suitable markers to estimate the relative contribution of breeding populations to a particular feeding ground (Avise and Bowen 1994, Norman et al. 1994).

Bass et al. (1996) used mtDNA sequence analysis to assess the genetic relationships among seven hawksbill nesting colonies in the tropical West Atlantic Ocean. This survey demonstrates significant mtDNA haplotype frequency shifts among most Caribbean nesting populations, and reveals a number of "endemic" haplotypes in sampled nesting aggregates. These strong population genetic partitions provide an appropriate basis for assessment of hawksbill feeding aggregates.

Here, we use mtDNA haplotype frequencies to resolve the demographic origins of a (primarily juvenile) feeding cohort at Mona Island, Puerto Rico. The scientific literature contains suggestions that hawksbill turtles may be nonmigratory or less migratory than other marine turtles (Carr 1952, Carr et al. 1966), but more recent studies have demonstrated at least occasional long-distance movements (Carr and Stancyk 1975, Meylan 1982, Parmenter 1983, Bjorndal et al. 1985), including a trans-Atlantic tag recovery (Marcovaldi and Filippini 1991). If the hawksbill turtle is relatively sedentary during post-pelagic life history, one expected consequence would be extensive sharing of mtDNA haplotypes between nesting areas and adjacent foraging areas. Alternatively, if the hawksbill turtle typically migrates between spatially distinct feeding and nesting habitat, this migratory behavior should be reflected in extensive mixing of mtDNA haplotypes (representing regional nesting populations) on foraging habitats. Hence, the most basic question addressed here is whether or not a feeding ground is composed primarily of turtles from adjacent nesting habitat. A more complex version of this question is whether or not the relative contributions of regional rookeries to a foraging habitat can be resolved with ML analysis.

The motivation for resolving stock composition intensifies as the conservation status of this species continues to deteriorate (National Research Council 1990). The abundance of *Eretmochelys imbricata* is greatly reduced relative to historical levels, and this species continues to be commercially harvested in the West Atlantic Ocean and Caribbean Sea (Carr and Meylan 1980, Canin 1989, Ottenwalder and Ross 1992). "Tortoiseshell" is the primary product of the hawksbill fishery, but eggs, meat, leather, and stuffed, mounted juveniles are also marketed in some areas (King 1982, Witzell 1983, Milliken and Tokunaga 1987). While $<10\,000$ nesting females may remain in the Caribbean (Meylan 1989), annual harvest in this area has exceeded 12 000 turtles (males, females, and juveniles) in recent years (Canin 1989). In view of this alarming depletion, the hawksbill turtle is considered to be in danger of extinction (Groombridge 1993) and has been placed on the World-Wide Fund for Nature (WWF) list of the 10 most endangered species.

Methods

Samples from feeding habitat around the southwest corner of Mona Island, Puerto Rico ($n = 41$) were collected during July and August 1993. Most (37 of 41) feeding-ground specimens were classified as juveniles, based on carapace length. The four captured adults included three males and one female. A small aliquot of blood ($\approx$1 mL) was removed from the cervical sinus of captured turtles, using the technique described by Owens and Ruiz (1980). Blood was stored in a lysis buffer [100 mmol/L Tris-HCl, pH 8; 100 mmol/L EDTA, pH 8; 10 mmol/L NaCl; 1.0% sodium dodecyl sulfate] in approximately a 1:10 ratio of blood to buffer, as recommended by White and Densmore (1992). Our experience indicates that blood collected for genetic analysis can be preserved in lysis buffer for extended periods (>1 yr) at room temperature without significant DNA degradation.

DNA isolations were conducted with standard phenol/chloroform methodology (Hillis and Moritz 1990). mtDNA control region sequences were amplified via polymerase chain reaction methodology (PCR; Mullis and Faloona 1987) using biotinylated versions of primers TCR-5 and TCR-6 (Norman et al. 1994). An 18-base "universal" M13 sequence was added to the 5′ end of primers to facilitate automated sequencing. The cycling thermal parameters used are as follow: 1 cycle at 94°C (3 min) followed by 35 cycles at 94° (1 min), 50° (1 min), and 72° (1 min). Standard precautions, including negative controls (template-free PCR reac-

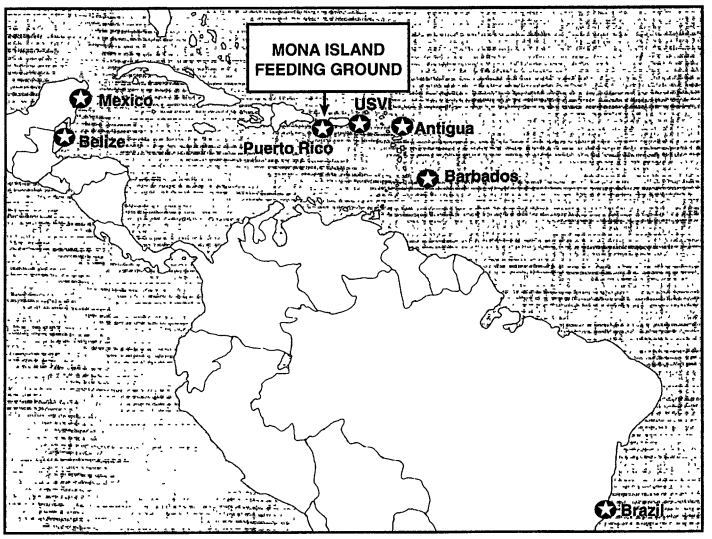

FIG. 1. Distribution of sampled nesting colonies for the Caribbean hawksbill turtle, following Bass et al. (1996). The Mona Island feeding ground and nesting colony are adjacent to the eastern coast of Puerto Rico.

tions), were used to test for contamination and to assure the fidelity of PCR reactions.

Streptavidin-coated magnetic beads (Dynabeads M280 streptavidin, Dynal, Sweden) were used to purify PCR products (Mitchell and Merrill 1989). Single-stranded template was generated by denaturing the magnetically captured double-stranded DNA with fresh 0.15 mol/L NaOH, and using the released (nonbiotinylated) strand as a template for sequencing reactions. Single-stranded sequencing reactions were conducted with fluorescently labelled M13 primers in a robotic work station (Applied Biosystems model 800), and the labelled extension products were analyzed with an automated DNA sequencer (Applied Biosystems model 373A) in the DNA Sequencing Core at University of Florida.

Control regions sequences from the Mona Island feeding ground were compared to a bank of sequences from rookery samples described in Bass et al. (1996). Rookery samples (eggs or blood aliquots from different females; $n = 14–15$ per location) include locations in Belize, Quintana Roo (Yucatan, Mexico), U.S. Virgin Islands, Antigua, Barbados (British West Indies), Bahia (Brazil), and Mona Island (Puerto Rico, USA) (Fig. 1). These locations represent most of the major nesting aggregates for *Eretmochelys imbricata* in the Caribbean.

Sequences that matched known haplotypes were collated for analysis, whereas new or ambiguous genotypes were sequenced in the opposite direction to assure accuracy. At least one representative for every haplotype was sequenced in both directions. Feeding area specimens that matched known haplotypes were assigned a letter code, following Bass et al. (1996). New haplotypes observed only in the feeding population were assigned Greek letters (alpha, beta, gamma) to distinguish them from known nesting-beach haplotypes.

The most basic test of demographic affinities involves a comparison of haplotype frequencies between the feeding populations and regional rookeries. In this study, the Mona Island feeding population was compared to regional rookeries using a G test of independence with Yates correction (Sokal and Rohlf 1981). Haplotype diversity was calculated following the approach of Nei (1987: Eq. 8.4).

To assess the relative contribution of regional nesting colonies to the Mona Island foraging area, we used an unconditional ML analysis (Pella and Milner 1987) in the DOS-compatible program UCON (Masuda et al. 1991). The unconditional ML approach is recommended when the presence of rare haplotypes may bias the outcome (J. Pella and M. Masuda, *personal communication*), but, in most cases, it yields qualitatively similar results to the conditional ML algorithm (Xu et al. 1994). Standard deviations on ML estimates were calculated with the "jackknife" option in UCON.

Broderick (1992) used the conditional maximum likelihood (ML) program GIRLSYM (Masuda et al. 1991) to evaluate the impact of sample size on the accuracy of mixed stock assessments, under conditions typical for regional (mtDNA) population structure in marine turtles. These simulations indicate that 30–40 specimens from a presumed mixed stock will yield reasonably accurate (±10%) estimates of feeding-ground composition under typical conditions. An important condition of this generalization is that most candidate nesting areas are characterized by at least a 30% frequency shift in mtDNA haplotype frequencies. In general, a higher frequency shift between candidate rookeries provides a more powerful test of mixed stock composition (Pella and Milner 1987, Xu et al. 1994).

RESULTS AND DISCUSSION

In this survey, we examined a 351 bp (base pairs, the fundamental units of double-stranded DNA) frag-

TABLE 1. Polymorphic sites observed in a 351 bp (base pairs) fragment of the mtDNA control region from 41 hawksbill turtles sampled in foraging habitat around Mona Island, Puerto Rico. Sequence numbering begins at the 3′ end of primer TCR-5 (Norman et al. 1994) and letter designations for haplotypes follow Bass et al. (1996), with new haplotypes indicated by greek letters (α, β, γ). For a complete reference sequence, see Bass et al. (1996). Asterisks (*) indicate two sites that were polymorphic in feeding ground samples but not in the companion nesting population survey.

Genotype letter code	Polymorphic sites									
	13	62	96	158	210	252*	255*	309	310	341
A	A	T	A	C	T	A	T	T	G	G
B	G	T	G	T	C	A	T	C	G	A
F	G	C	G	T	C	A	T	C	G	A
L	G	C	A	T	C	A	T	C	A	A
N	G	C	G	T	C	A	T	C	A	A
Q	G	C	A	T	C	A	T	C	G	A
α	A	T	A	C	T	A	C	T	G	G
β	A	C	A	T	C	A	T	G	G	A
γ	G	C	A	T	C	G	T	C	G	A

ment of the mtDNA control region [matching sites 10–360 in Bass et al. (1996)] that contains all of the polymorphic sites reported in the nesting-population survey of 384 bp. Ten polymorphic sites were observed in foraging-area samples (Table 1), including eight of the 17 variable positions reported for surveyed nesting colonies and two additional polymorphic sites. These polymorphic sites define nine haplotypes (Table 2): six haplotypes that match sequences described in the nesting-colony survey and three new haplotypes. Notably, the six haplotypes that match nesting colony haplotypes account for 37 of 41 samples, and include the four most abundant haplotypes reported in the nesting-population survey. Haplotypic diversity for Mona Island foraging samples is relatively high ($h = 0.739$) compared to the range of values reported in the nesting-beach survey ($h = 0.125$–0.782; Bass et al. 1996).

The simplest assessment of the relationship of feeding populations to nesting populations involves a comparison of the Mona Island rookery to the Mona Island foraging area. A G test of independence demonstrates that the feeding population is significantly different from the adjacent nesting population ($P < 0.005$) and from all other assayed nesting sites ($P < 0.01$). From this information, we conclude that the Mona Island feeding population is not drawn primarily from the proximal nesting colony or from any single assayed nesting colony. Whereas more convoluted explanations may be invoked to explain these results (such as unsurveyed rookeries in the vicinity of Mona Island), the simplest explanation entails immigration from multiple nesting populations.

This conclusion is supported, in a qualitative fashion, by the presence of haplotypes in feeding ground samples that are observed only in single assayed nesting colonies. Specimens from the Mona Island foraging area contain haplotypes B ($n = 1$), L ($n = 1$), N ($n = 3$), and Q ($n = 7$), observed only in Antigua (B), Mona Island (L and N), and Yucatan (Q) nesting areas, respectively (Table 2). Whereas some of these haplotypes eventually may be detected in other nesting colonies, the overall pattern is consistent with the hypothesis that foraging aggregates are composed of turtles from nesting habitats throughout the region.

The ML analysis indicates that five of the seven sampled rookeries contribute to this feeding population at levels greater than zero, with the largest contributions from the U.S. Virgin Islands and the Yucatan Peninsula (Table 3). However, additional nesting populations may also contribute to these feeding grounds. Rookeries are known from the Dominican Republic, Cuba, and elsewhere, but samples were unavailable from these locations due to logisitic limitations, permit problems, or political considerations. Many diminutive nesting col-

TABLE 2. Distribution of haplotypes on seven nesting areas and the Mona Island feeding ground. Most (37 of 41) feeding ground samples are juveniles. Among the four adult samples, one male contained haplotype Q, two males contained haplotype N, and one female contained haplotype F. See Bass et al. (1996) for details about sample locations.

Location	Haplotype																					
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	α	β	γ
Belize						11	1	1	1															
Yucatan																2	13							
Mona Is.	1					1				2	1	1	2	6	1									
Virgin Is.	1					14																		
Antigua	9	4	2																					
Barbados	11			1	3																			
Bahia, Brazil	4																	6	2	1	1			
Mona Island foraging habitat	7	1				18						1		3			7					2	1	1

TABLE 3. Contribution of assayed nesting colonies to the coastal feeding area at Mona Island, Puerto Rico, as indicated by unconditional maximum likelihood analysis. The four surveyed individuals that contained haplotypes not observed in nesting populations were removed from the analysis. Standard deviations were calculated with the infinitesimal jackknife option in the program UCON (Masuda et al. 1991).

Location	Contribution	SD
Gale's Point, Belize	0.021	0.004
Quintana Roo, Mexico	0.189	0.064
Mona Is., Puerto Rico	0.127	0.058
Buck Island, U.S.V.I.	0.510	0.090
Jumby Bay, Antigua	0.121	0.071
Barbados	0.030	0.041
Bahia, Brazil	0.000	0.000

onies exist throughout the West Atlantic, and if one considers the beaches where only a handful of hawksbill turtles nest, there may be dozens of unsampled locations. Hawksbill turtles are less colonial in their nesting habits than other sea turtles, and this diffuse nesting is refractory to conventional sampling strategies. However, in considering the geographic breadth of (genetically defined) nesting populations, the presence of unsampled nesting beaches may not be an overwhelming limitation. Broderick et al. (1994) reported that Indo-Pacific nesting beaches separated by several hundred kilometres were not significantly different in terms of mtDNA frequency distribution, consistent with tagging studies demonstrating that hawksbill turtles may move between adjacent nesting habitats (Diamond 1976, Limpus et al. 1983). Hence, when one nesting beach is sampled for mtDNA analysis, it probably represents an extended nesting population including several adjacent nesting sites.

We regard the results of the ML analysis as general *qualitative* indicators of the contribution to Mona Island feeding habitat from regional nesting populations. Although the specific contributions of surveyed rookeries may not be precisely resolved, the ML analysis nonetheless provides clues as to the geographic scale of recruitment to the Mona Island foraging area. This feeding population evidently is not drawn exclusively from the neighboring (Mona Island) rookery, and is not drawn at detectable levels from the surveyed South Atlantic (Bahia, Brazil) nesting population. Hence, the ML analysis indicates that turtles recruit to this feeding population on a scale >100 km but less than the 7000 km that separate Mona Island from Bahia, Brazil. We suggest, as a first approximation, that this feeding population contains cohorts from throughout the Caribbean region.

The conclusion of mixed-stock composition in Caribbean feeding grounds is consistent with results of the survey of mtDNA haplotypes in Indo-Pacific hawksbill populations; Broderick et al. (1994) concluded that a foraging aggregate in the Torres Strait region of northern Australia is occupied by juveniles from several nesting populations. Indeed, results of this study, along with feeding-ground surveys of green turtles (Carr 1975, Limpus et al. 1992) and loggerhead turtles (Laurent et al. 1993, Sears et al. 1995), indicate that overlap of nesting populations in regional feeding areas may be a general feature of marine turtle population biology.

The presence of hawksbill turtles on distant feeding areas demonstrates the complex nature of marine turtle conservation. A nesting population within the boundaries of one nation can be diminished by a harvest in a neighboring (or even a distant) nation. The Caribbean hawksbill trade has been active in recent years, with Cuba, Haiti, and Panama being the largest exporters (Canin 1989, Ottenwalder and Ross 1992) and Japan being the largest importer (Milliken and Tokunaga 1987). This international commerce was discontinued in December 1992, but a local trade flourishes in many locations and there is continuing interest in reopening the international trade (M. Donnolly, *personal communication*). In light of the results obtained here, the organized harvest of adults and juveniles on coastal feeding grounds (see Ottenwalder and Ross 1992) may be especially damaging, as this type of fishery will diminish reproductive populations throughout the region.

Clearly, mtDNA markers can be a powerful tool for assessing anthropogenic impact on migratory cohorts (Bowen et al. 1995, Sears et al. 1995), and it is apparent that nuclear DNA assays will provide additional markers for demographic assessment (Karl et al. 1992, FitzSimmons et al. 1995). In the parlance of international conservation, genetic markers allow wildlife agencies to identify "range states," nations impacted by the harvest of natural resources at a distant location. The concept of range states implies some level of jurisdiction: the 1982 U.N. Convention on the Law of the High Seas recognizes that nations hosting the developmental habitat for migratory marine species hold exclusive fishing rights for these animals on the high seas (Van Dyke 1993). The 1983 U.N Convention on the Conservation of Migratory Species (a.k.a. the Bonn Convention) prohibits taking endangered species during migrations on the high seas (Hykle 1992). The principles outlined in these conventions, as applied to sea turtles, provide nations that host nesting and developmental habitats with some level of jurisdiction over these animals on geographically remote feeding grounds. Genetic tags can play a critical role in identifying the origins of beleaguered marine turtle feeding populations (Bowen 1995, Bowen and Avise 1995), and eventually may provide a foundation for international agreements concerning the stewardship of migratory marine species.

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Note Added in Proof

Recent research has demonstrated that four mtDNA haplotypes observed in the Brazilian nesting population (R, S, T, and U in Table 2) may be derived from Atlantic loggerhead turtles (*Caretta caretta*). Available evidence, including morphological examination (P. C. H. Pritchard, *personal communication*) and nuclear DNA assays (S. A. Karl, *personal communication*), indicate that these samples are not misidentified loggerheads or first-generation hybrids, but the product of an older introgression event (Bass et al. 1996). While it is uncertain how the introgression of loggerhead genotypes could affect hawksbill migratory behavior, these findings do not alter the conclusion that the Brazilian nesting colony did not contribute to the assayed feeding aggregate at detectable levels.